IN-VITRO AND MOLECULAR DOCKING STUDIES OF SEVEN SELECTED MYRTACEAE PLANTS FOR NEURAMINIDASE ACTIVITY

by

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LIST OF SYMBOLS AND ABBREVIATIONS

µg	Microgram
µM	Micromolar
^{13}C NMR	Carbon Neuclear magnetic Resonance
1D NMR	One Dimentional Neuclear magnetic Resonance
^{1}H NMR	Proton Nuclear magnetic Resonance
^{1}H-^{13}C HMBC	Heteronuclear Multiple-Bond Correlation
^{1}H-^{13}C HSQC	Heteronuclear Single-Quantum Correlation
^{1}H-^{1}H COSY	Homonuclear Correlation Spectroscopy
2D NMR	Two Dimentional Neuclear magnetic Resonance
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
BuoH	Butanol
CADD	Computer aided drug design
CC	Column chromatography
$CHCl_3$	Chloroform
d	Doublet
dd	Doublets of doublet
ddd	Doublets of doublet of doublet
DEPT NMR	Distortionless Enhancement by Polarization Transfer
EtoAc	Ethyl acetate
FT-IR	Fourier Transform Infrared
Glu	Glutamic acid
Gly	Glycine
HA	Hemagglutinin.
HS-GC/MS	HeadSpace Gas Chromatography Mass Spectrometry
Hz	Hertz

IC_{50}	Half-maximal inhibitory concentration
Lys	Lysine
m	Multiplet
m/z	Mass/charge
M1	Matrex
M2	Ion chanel
MES	2-(N-Morpholino)EthaneSulfonic Acid
Methanol-*d*5	Deuterated Methanol
mg	Milligram
MHz	Mega Hertz
MS	Mass spectrscopy
MUNANA	2′-(4-methylumbelliferyl)-α-d- N-acetylneuraminic acid
NA	Neuraminidase
NAI	Neuraminidase inhibitor
Neu5AC	N-acetylneuraminic acid
NP	nucleoprotein
PA	Viral RNA polymerase
PB1	Viral RNA polymerase
PB2	Viral RNA polymerase
PDB	Protein Data Bank
PTLC	Preparative Thin Layer Chromatography
Pyridine- *d*5	Deuterated Pyridine
s	Singlet
SEMI-PREP HPLC	Semi Preparative High Performance Liquid Chromatography
Ser	Serine
SPE	Solid phase microextraction
t	Triplet
TLC	Thin Layer Chromatography
Tyr	Tyrosine
v/v	Volume/volume

w/v	Weight/volume
δ	Chemical shift

KAJIAN *IN- VITRO* DAN PENDOKAN MOLEKUL TUJUH TUMBUHAN MYRTACEAE TERPILIH UNTUK AKTIVITI NEURAMINIDASE

ABSTRAK

Virus influenza A menyebabkan masalah kesihatan yang teruk kepada manusia dan beberapa spesies haiwan yang boleh membawa kepada morbiditi dan kematian dalam kalangan pesakit yang dijangkiti. Oseltamivir (Tamiflu) dan zanamavir (Relenza) adalah dua drug perencat NA terkini yang telah diluluskan oleh Pentadbiran Makanan dan Drug (FDA, USA). Walaubagaimana pun, oleh kerana kemunculan rintangan virus terhadap kedua-dua drug ini, terdapat keperluan mendesak untuk menemui suatu perencat neuraminidase alternatif. Kajian ini telah dijalankan untuk menyiasat aktiviti perencatan neuraminidase tujuh tumbuhan terpilih daripada keluarga Myrtaceae: *P. guajava*, *S. cumini*, *S. grande*, *S. jambos*, *S. malaccense* jambu bol, *S. samaranges, dan S. malaccense* jambu susu. Tumbuh-tumbuhan telah diekstrak menggunakan metanol dan difraksi menggunakan *n*-heksana, kloroform, etil asetat dan butanol. Minyak pati daripada daun tujuh terpilih ini telah diasingkan menggunakan hidropenyulingan dan dianalisis dengan kromatografi-spektrometri jisim. Ekstrak mentah, fraksi, sebatian dan minyak pati telah diuji terhadap assai enzim neuraminidase *Clostridium perfringens* dan H1N1. Saringan maya menggunakan Autodock Vina terhadap tapak aktif protein neuraminidase H1N1 (kod PDB: 3TI6) telah digunakan untuk menyaring sebatian-sebatian yang dikenal pasti daripada minyak pati, dan kemudian sebatian-sebatian yang dikenal pasti diubahsuai dengan menambahkan kumpulan fungsi yang ada pada oseltamivir iaitu drug piawai. Pemencilan telah dilakukan dari fraksi etil asetat (daun

dan buah-buahan) daripada *P. guajava* untuk menghasilkan satu sebatian baru, 1,3-benzotiazol-asid oksalik dan lima sebatian yang telah dikenali, asid kojik, asid galik, kuersetin, asid ursolik dan ursolik aldehid. Kebanyakan daripada ekstrak mempamerkan perencatan yang bagus, buah *P. guajava* 89% dan tujuh daun yang menpunyai kadar perencatan antara 70 hingga 89% bagi *C. perfringens.* Bagi fraksi yang lain, perencatan antara 12.2 hingga 97% terhadap NA daripada *C. perfringens* sementara 12.6 hingga 97.9% terhadap NA daripada H1N1. Tiga pati minyak menunjukkan perencatan tinggi sehingga 57.8, 88.7 dan 91.2% dalam kalangan tujuh pati minyak. IC_{50} bagi dua sebatian terpencil, kuersetin adalah 229 µM, asid galik adalah 187 µM dan DANA 4.3 µM untuk NA bakteria, dan kuersetin 127 µM, asid galik 258 µM dan DANA 3.9 µM untuk NA virus H1N1. Pelbagai sebatian utama telah dikenalpasti daripada setiap varieti adalah 20.7% α-pinena daripada *P. guajava,* 25.1% Z-β-osimena daripada *S. cumini,* 33.1% δ-terpinena daripada *S. samarangense,* (Z)-3-heksanol 23.5% daripada *S. malaccense* jambu susu dan 62.5%, 20.5% dan 23.0% β-kariofilena masing-masing daripada *S. grande, S.malaccense* jambu bol dan *S. Jambos*. Pendokan molekul *in silico* sebatian terpencil daripada *P. guajava* dan sebatian yang dikenal pasti daripada minyak pati aktif telah dikaitkan dengan keputusan eksperimen. Semua sebatian mempamerkan interaksi dengan protein sasaran pada dua tapak mengikat berbeza, iaitu tapak aktif konservatif dan poket bersebelahan (kaviti 150). Hubungan antara *in silico* dan keputusan eksperimen menunjukkan bahawa aktiviti yang diperolehi bukan disebabkan oleh sebatian tunggal tetapi dari kesan sinergi, yang mana semua sebatian menyumbang untuk merencat neuraminidase. Kesimpulannya ekstrak mentah, fraksi, tiga daripada tujuh minyak

pati tumbuh-tumbuhan terpilih menunjukkan perencatan neuraminidase yang baik dan boleh menjadi sumber perencat neuraminidase.

IN-VITRO AND MOLECULAR DOCKING STUDIES OF SEVEN SELECTED MYRTACEAE PLANTS FOR NEURAMINIDASE ACTIVITY

ABSTRACT

Influenza A virus cause severe health problems to humans and several animal species which could result in morbidity and mortality in infected patients. Oseltamivir (Tamiflu) and zanamivir (Relenza) are two current drug used NA inhibitors that have been approved by the Food and Drug Administration (FAD, USA). However, due to the emergence of virus resistance to these drugs, there is an urgent need to discover an alternative neuraminidase inhibitors. The present study was undertaken to investigate the neuraminidase inhibition activity of seven selected plants that belong to the family of Myrtaceae; *Psidium guajava, Syzygium cumini, Syzygium grande, Syzygium jambos, Syzygium malaccense* cultivated jambu bol, *Syzygium samaranges,* and *Syzygium malaccense* cultivated jambu susu. The plants were extracted using methanol and fractionated using *n*-hexane, chloroform, ethyl acetate and butanol. Essential oils from leaves of seven selected plants were isolated by hydrodistillation and analyzed by head-space chromatography–mass spectrometry. The crude extract, fractions, compounds and essential oil were subjected to neuraminidase inhibition assay against *Clostridium perfringens* and H1N1 neuraminidase enzyme. Molecular docking using Autodocktools 4.2 were applied against the active site of neuraminidase H1N1 protein (PDB code: 3TI6) for the isolated and identified compounds from essential oil. The isolation was done from the ethyl acetate fraction of (leave and fruit) of *P. guajava* to yield one new compound 1,3-benzothiazole-oxalic acid and five

known compounds kojic acid, gallic acid, quercetin, ursolic acid and ursolic aldehyde. Good inhibition exhibited by most of the extracts, *P. guajava* fruit 89% and seven leaves ranging from 70 to 89% against *C. perfringens*. For the other fractions the inhibition ranged from 12.2 to 97% against NA from *C. perfringens* while 12.6-97.9% against NA from H1N1. Three essential oils showed high inhibition up to 57.8, 88.7 and 91.2% among the seven oils. The IC_{50} of two isolated compounds quercetin was 229 µM, gallic acid was 187 µM and DANA 4.3 µM for the bacterial NA, and quercetin was 127 µM, gallic acid was 258 µM and DANA 3.9 µM for the NA H1N1 virus. Different major compounds identified in essential oil from each variety were 20.7% α-pinene in *P. guajava,* 25.1% *Z*-β-ocimene in *S. cumini*, 33.1% δ-terpinene in *S. samarangense*, (*Z*)-3-hexanol (23.5%) in *S. malaccense* cultivated jambu susu and 62.5%, 20.5% and 23.0% β-caryophyllene in *S. grande, S. malaccense* cultivated jambu bol and *S. Jambos,* respectively. The *in silico* molecular docking of isolated compounds from *P. guajava* and identified compounds from active essential oil were correlated with the experimental results. All the compounds exhibited interactions with the target protein at two different binding sites, namely, conservative active site and adjacent pocket (150-cavity). The correlation between the *in silico* and experimental results revealed that the obtained activity was not due to a single compound but from a synergistic effect, where all the compounds contributed to inhibit neuraminidase. In conclusion crude extracts, fractions, three out of seven essential oils of selected plants showed good neuraminidase inhibition and could be source of neuraminidase inhibitors.

CHAPTER ONE

INTRODUCTION

1.1 The influenza A viruses

The human-human transmission rate of the influenza virus A (H1N1) virus and their effects on human health attracted global attention in 2009. Influenza A viruses are isolated from numerous host species, such as marine mammals, horses, pigs, domestic birds, humans, and dogs (Webster *et al*., 1992). Influenza A viruses belong to the *Orthomyxoviridae* family and cause infrequent epidemics that usually result in increased death rates compared with seasonal influenza epidemics. These viruses have an RNA genome consisting of eight gene segments (Knipe *et al*., 2007; Wright *et al*., 2007) with two neuraminidase (NA) and a hemagglutinin (HA) as surface glycoproteins in the envelope (Lamb *et al*., 2001; Wright *et al*., 2001). Influenza A viruses are sorted into antigenic HA and NA subtypes. A total of 16 antigenic HA (H1–H16) and 9 NA (N1–N9) subtypes have been confirmed. H1N1, also called swine flu, consists of an H1 subtype HA and an N1 subtype NA (Schwahn *et al.*, 2010). H3N2, H2N2, and H1N1 are considered the most important influenza A subtypes that infect humans during seasonal epidemics (Taubenberger *et al*., 2005). Influenza A viruses contain a genome consisting of eight segments involving a negative-sense, single-stranded RNA that encodes one or two proteins Figure 1.1.

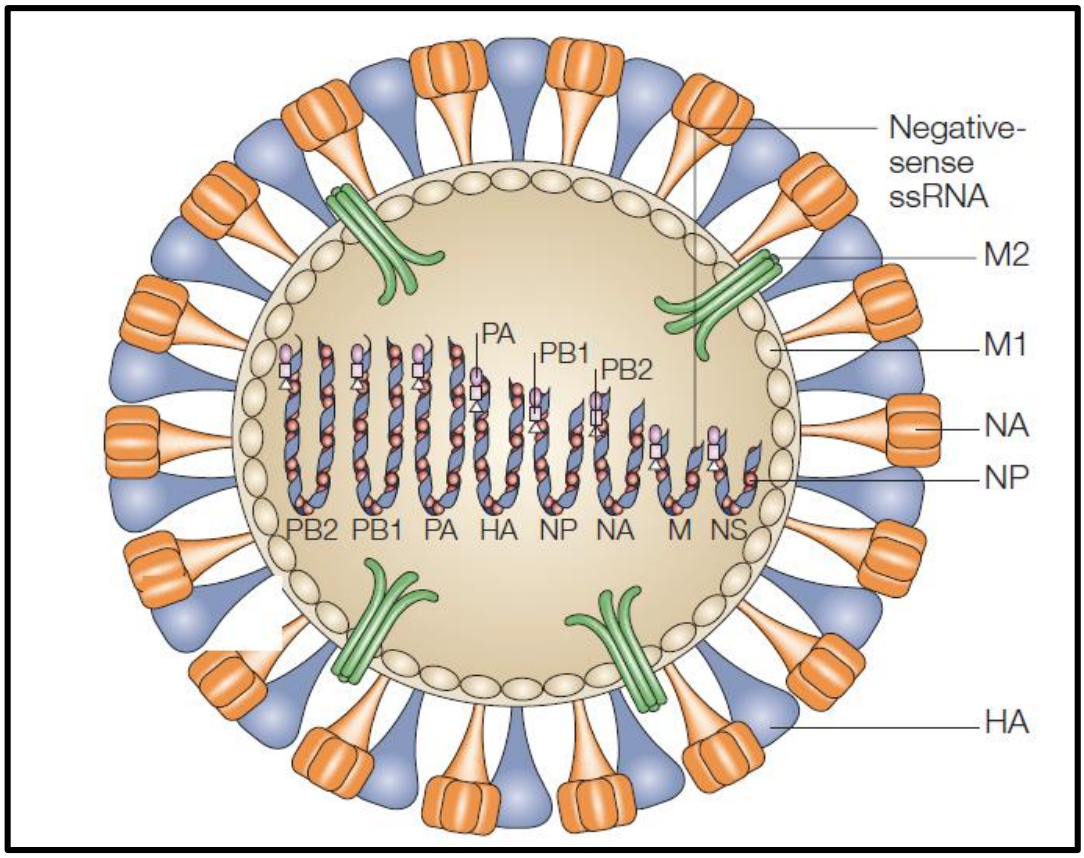


Fig. 1.1 Schematic diagram of influenza A viruses (Horimoto and Kawaoka, 2005)

1.2 Influenza pandemics

Influenza viruses cause global pandemics as well as repeated epidemics. In the 20th century, three major worldwide pandemics of influenza occurred at different interval in 1918, 1957, and 1968 (Taubenberger and Morens, 2006; Kilbourne, 2006). Spanish flu in 1918 was the most devastating pandemic that caused the death of 50 million people (Johnson and Mueller, 2002). The subsequent epidemics in 1957 occurred in Asia and Hong Kong flu pandemic of 1968; these two epidemics were milder, together caused a total of approximately two million deaths (Kilbourne, 2006). An important number of infected people would be detected resulting from the recently emerged H1N1 influenza virus and spread in many worldwide locations. In the

southwestern United States and Mexico 2009 new influenza A (H1N1) has been reported (Pan *et al*., 2010). During the epidemic 2009 H1N1 the new strain of swine flu (hosted by pigs) attracted the attentions of most of the organizations, because this epidemic gain the ability of transfer from human to human.

1.3 Swine influenza virus (SIV)

A global influenza preparedness has been developed by World Health Organization (WHO), this plan used to define the pandemic stages which can be used as national measures before and during the pandemic. Concerning the swine influenza (H1N1) pandemic, the WHO raised alert levels according to the different H1N1 phases, namely phases 4, 5, and 6, on 27 April 2009, 29 April 2009, and 11 June 2009 respectively, these grouped into six-phases, phase 1-3 correlate with preparedness these including the capacity development and the response planning activities, while or phases 4-6 gave clear signal the need for serious response and more mitigation efforts.. Afterward, the WHO declared a full-blown influenza pandemic. Laboratories showed genuine cases of H1N1 reported from more than 214 countries in June 2010, with the casualties reaching 18,156 (WHO, 2009).

The pandemic H1N1 virus that spread in 2009 comprised various re-assorted viral genes from different sources. The virus was introduced to populations in 1998 from the avian virus with a North American lineage; this H1N1 virus consisted of two polymerase genes, eight segmented genomic RNAs, PA, and PB2 (Krause μμ., 2010). Two different surface glycoproteins, NA and HA (Lamb and Krug, 2001; Wright and Webster, 2001), have important protective immunity within a host (Garten *et al*.,

2009). This unique genetic combination may contribute to the improved fitness of the H1N1 in humans and its human-to-human transmissibility, No molecular functions have been shown before to confer the increased human-to-human transmissibility of the 2009 H1N1.

1.4 Life cycle of influenza A virus

The HA protein is critical for binding cellular receptors and fusing viral and endosomal membranes (Figure 1.2) (Das *et al*., 2010)**.** The life cycle of the influenza A virus is divided into different stages: (1) viral entry to the host cell, (2) entry of viral ribonucleoprotein complex (vRNP) into the nucleus, (3) transcription and replication of the viral genome, (4) vRNP exportation from the nucleus (5) assembly, and then (6) budding at the plasma membrane of the host cell (Samji, 2009).

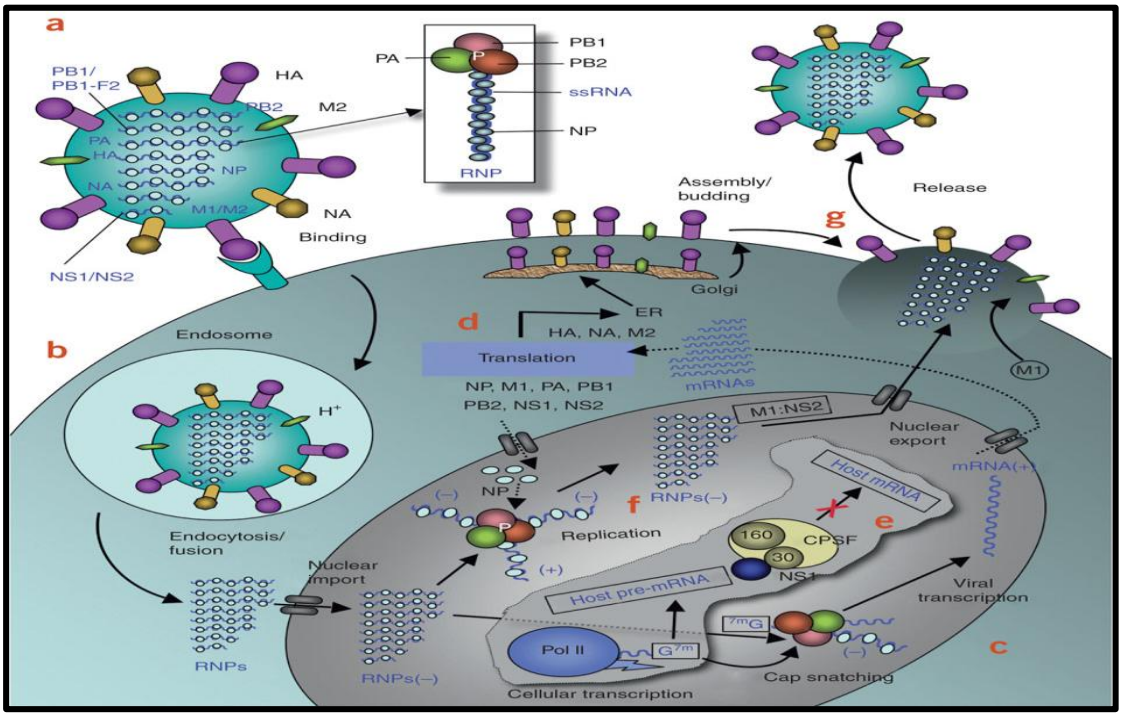


Fig. 1.2 Schematic diagram of the influenza viral life cycle (Das *et al*., 2010).

Influenza is spread via tiny droplets expelled through coughing or sneezing, thus can be easily inhaled thereby transmitting the disease. Once the virus has entered the host within the respiratory tract, particularly in the nose, mouth and throat, it will fuse to the plasma membrane of permissive cells lining in the respiratory tract. The projecting spikes on the viral lipid membrane known as hemagglutinin (HA) bind to sialic acid (SA) of the host cell's membrane. Two major linkages between SA and the carbohydrates which are important for the HA specificity is the α (2, 3) and α (2, 6) found in different species (Samji, 2009). Humans influenza recognize α (2, 6) linkages, avian influenza; α (2, 3) linkages while swine influenza recognize both linkages (Skehel and Wiley, 2000). This explains the importance of swine being a good mixing vessel for avian and human influenza viruses, hence producing dangerous pathogenic viruses.

After the attachment, receptor mediated endocytosis occurs and the virus is engulfed by the cell in an endosome at low pH of around 5 to 6. The acidic environment of the endosome induces conformational change in HA, bringing both the viral and endosomal membranes into contact with each other (Skehel and Wiley, 2000; Huang *et al*., 2003) and also opens up the M2 ion channel that acts as a proton selective ion channel, thus acidifies the viral core (Pinto and Lamb, 2006; Holsinger *et al*., 1994).

The viral constituents (vRNP) will then be released into the cytoplasm and migrate through the cytoplasm to the cell's nucleus. Viral transcription and replication occurs in the nucleus. The influenza viral genome is made up of negative sense strands of RNA and converted into a positive sense RNA for the genome to be transcribed.

The virus replicates its genome and forms new gene particles (Samji, 2009). New genes and proteins then migrate from the nucleus through the cytoplasm and to the cell membrane. The newly fanned viral begin to bud through the host cell's plasma membrane to form viral particles and eventually released into the extracellular environment to invade new cells (Scheiffele *et al.*, 1999). The new viral particles are coated with sialic (neuraminic) acid (SA), the same substance that bound them to the exterior of the cell they had invaded (Samji, 2009; Scheiffele *et al.*, 1999). The nascent viruses would stick to the membrane of the cell in which they had been formed, thus, unable to separate and travel to other cells in the respiratory tract.

The HA spike of one virus could stick to the SA coating of another, thereby causing viral aggregation and impeding the capability of the viruses to spread to, and invade, new cells. However, NA molecules, which are also part of the wrapping on the newly formed viral particles, cleave viral SA, thereby freeing the viruses from the host cell and from each other (Samji, 2009; Von Itzstein and Thomson, 2009). Drug that blocked the action of viral NA would thus prevent the escape of newly formed viruses from the host cells and thus preclude viral spread to other cells. This is the main reason as to why NA is taken as a drug target for developing agents in influenza virus drug discovery.

1.5 Sialic acid

Sialic acid (SA) is a common term for nine-carbon acidic amino sugars (5-amino-3,5-dideoxy-*D*-glycero-*D*-galacto-nonulosonic acid). Two new compounds, namely, *N*-glycolylneuraminic acid and *N*-acetylneuraminic acid (Neu5Ac), are obtained by substituting the *N*-glycolyl group and *N*-acetyl with the amino group in

SA (Figure 1.3). The hydroxyl group can be substituted with phosphate, methyl, sulfate, or lactoyl residues (Suzuki *et al*., 2000). Human influenza viruses preferably bind to the sialic acid-α2,6-galactose SAα2,6Gal linkage, whereas equine and avian influenza viruses preferably bind to the (SAa2,3Gal) linkage (Connor *et al*., 1994).

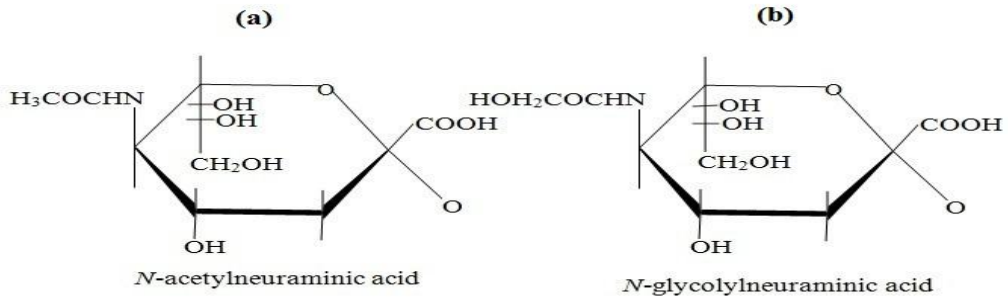


Fig. 1.3 Structure of (a) *N*-acetyl and (b) *N*-glycolylneuraminic acids. These SA differ at position 5 of the pyranose ring. *N*-Acetylneuraminic acid is the precursor of *N*-glycolylneuraminic acid; enzymatic hydroxylation of the former results in the latter.

1.6 Neuraminidase NA

Influenza A viruses carry two surface glycoproteins, namely, HA and NA. Both proteins recognize the same host cell molecule, that is, SA and Neu5Ac, which are found as the terminal carbohydrate unit of the upper respiratory tract and lung-associated glycoconjugates (Von Itzstein and Thomson, 2009). NA encodes six RNA segments and is known as the second major surface antigen of virions. NA acts as a pair of biological scissors clipping Neu5Ac residues, thus facilitating movement and viral release (Von Itzstein and Thomson, 2009). The NA morphology can be divided into three domains: the cytoplasmic domain of six amino acids, the *N*-terminal hydrophobic domain, and the actual stalk domain (Colman, 1994). The cytoplasmic domain contains six amino acids completely conserved in the NA of influenza A. This

condition is essential for NA inclusion into virions and for the assembly of the influenza A virus.

Scientists have found nine different NAs (N9). However, only N1 and N2 are important in human infection, whereas the other NAs have only been found in seven animals. Neuraminidase is the major surface protein of the influenza virus that extracellularly acts by cleaving the terminal neuraminic acid from cellular receptors recognized by the HA. According to phylogenetic studies, the neuraminidase of the influenza virus is divided into two groups. Group one consist of N1, N4, N5, and N8, whereas the other group consist of N2, N3, N6, N7, and N9 (Gong *et al*., 2007).

The neuraminidase plays an extremely important role in the pathogenesis of viral infection. That is, it facilitates the release of newly formed virions from the host cell surface to the neighbor cells that facilitated the spread of the virus. NA acts as a receptor-destroying enzyme in the influenza virus by removing the terminal SA of a viral receptor (De Clercq, 2006).

1.7 Neuraminidase inhibitors (NAIs)

Inhibitors block the active site of the neuraminidase and then prevent the release and spread of new virions. After infection, the patient should take an antiviral drug during the early stage if possible to achieve an effective treatment. Antiviral drugs are important role in the management of infection caused by the seasonal influenza virus. Adamantanes (M2 ion channel blockers) and NA inhibitors (NAIs) are two classes of antiviral drugs licensed to prevent and treat infections caused by the influenza A virus (Moscona, 2008). The first inhibitor analog of SA is 2,3-

didehydro-2-deoxy-*N*-acetylneuraminic acid or Neu5Ac2en (DANA) Figure 1.4 (Meindl *et al*., 1974).

The only two drugs approved by the Food and Drug Administration, oseltamivir and zanamivir currently effective for the treatment of influenza infections, the seasonal influenza virus, and the pandemic H1N1 virus. Recently, peramivir has been developed as a new neuraminidase inhibitor administered intravenously (Babu *et al*., 2000) Figure 1.4. It is currently approved for emergency use in Japan and Korea. In other countries, this drug is still undergoing clinical trials. Peramivir is based on DANA, but it contains a cyclopentane ring and has the features of both oseltamivir and zanamivir Figure 1.4. Yamashita *et al*. (2010) found a new potent NA inhibitor, laninamivir considered as a second-generation acts as a long-acting NA inhibito. Administered as single inhaled dose, laninamivir is found to be effective against the wild-type strain of the influenza A and B viruses and the oseltamivir-resistant H274Y mutations of seasonal H1N1 viruses, namely, H5N1 and H1N1 (Sugaya and Ohashi, 2010; Nguyen *et al*., 2010; Yamashita, 2010).

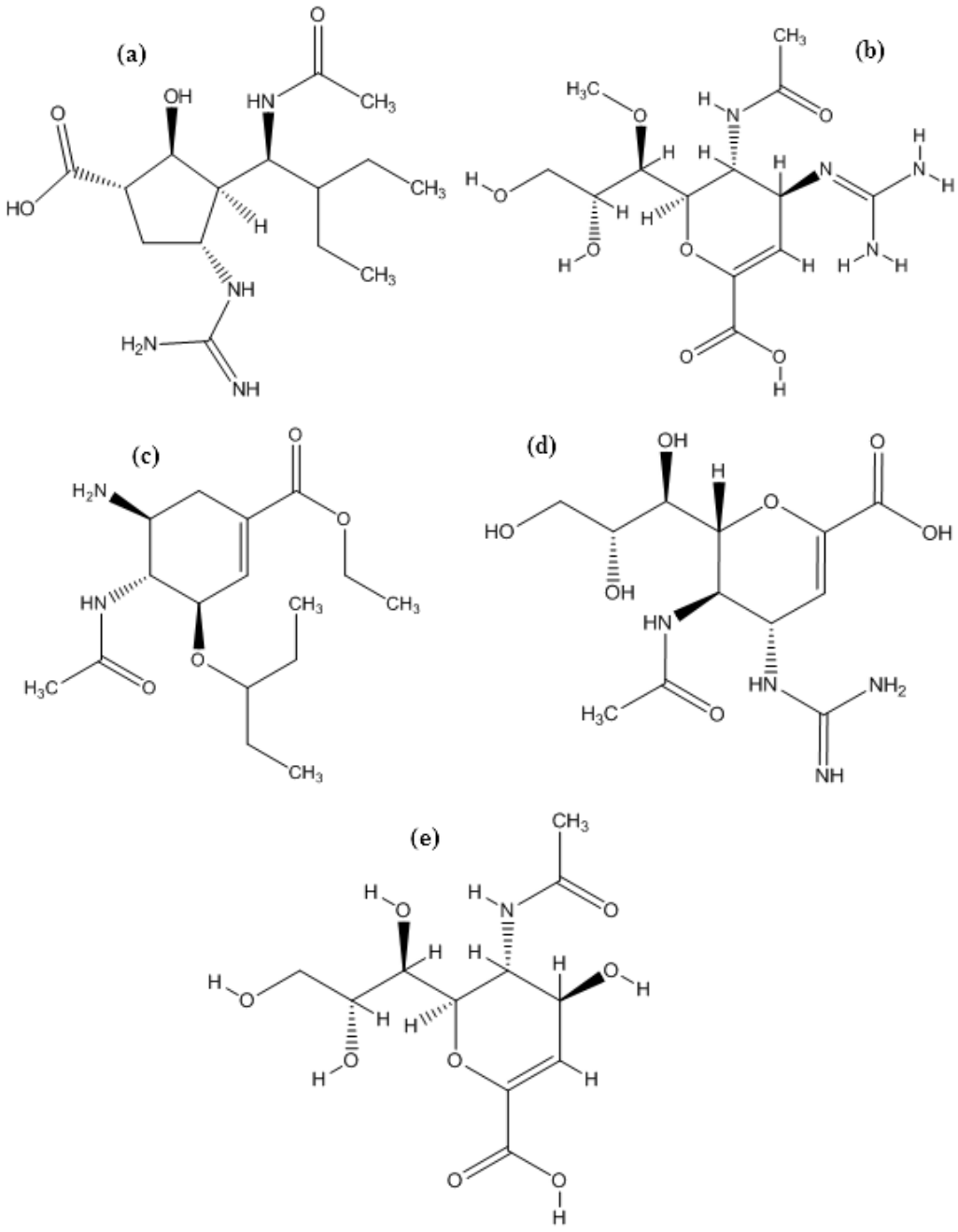


Fig. 1.4 Structures of (a) peramivir, (b) laninamivir, (c) oseltamivir, (d) zanamivir, (e) DANA.

1.8 Neuraminidase Active Site

The common feature among all influenza NAs is the active site Figure 1.5; with well formed large and rigid pockets form, can be considered as a common feature

among influenza NAs. SA and neuraminidase with conserved amino acids interact in a similar fashion with both substrate and inhibitor molecules (Xu *et al*., 2008; Kim *et al*., 1999). Therefore, antiviral drugs affecting the spread of viral replication have been developed.

The presence of four hydrophobic residues and ten Arg, Asp, and Glu residues make the neuraminidase active site highly polar (Von Itzstein and Thomson, 2009; Zhang and Xu, 2006). The active site in NA is divided into five regions from S1 to S5. S1 includes Arg 118, Arg 292, and Arg 371, which all bind to the carboxylate of substrate. S2 consists of Glu 119 and Glu 227, which are acidic residues responsible for forming the negative-charge region of the active site (Zhang and Xu, 2006). The hydrophobic region present at S3 forms at the side chains of Ile 222 and Trp 178, which are adjacent to the polar region of the Arg 152 side chain, and interacts with water molecules (Zhang and Xu, 2006; Russell *et al*., 2006). S4 is primarily a hydrophobic region derived from the side chains of Ile 222, Ala 246, and Arg 224. S5 has a mixed polarity and comprises of Ala 246 methyl and Glu 276 carboxylate that forms a hydrogen bond with the substrate hydroxyl group (Zhang and Xu, 2006).

The joining pockets of eight highly conserved amino acid residues are linked together, thereby causing the NA active site of these amino acids to directly come into contact with Neu5Ac and its derivatives (Von Itzstein, 2007). The active site is divided into three major pockets according to the crystal structure. Pocket 1 is formed by Glu 276, Glu 277, and Asn 294, which interact with the glycerol moiety of SA (Zhang and Xu, 2006). Pocket 2 forms a hydrophobic pocket that is not utilized by sialic acid for binding (Kim *et al*., 1997) and comprises the Ala 246, Ile 222, and Arg 224 side chains. Interactions with potential inhibitors are provided by these residues

because they are highly conserved. Pocket 3 is the largest pocket consisting of numerous amino acids, such as Glu 119, Asp 151, Arg 152, Trp 178, Ser 179, Th 222, and Glu 227, all of which interact with the N-acetyl and C-4 hydroxyl groups of sialic acid (Kim *et al*., 1997).

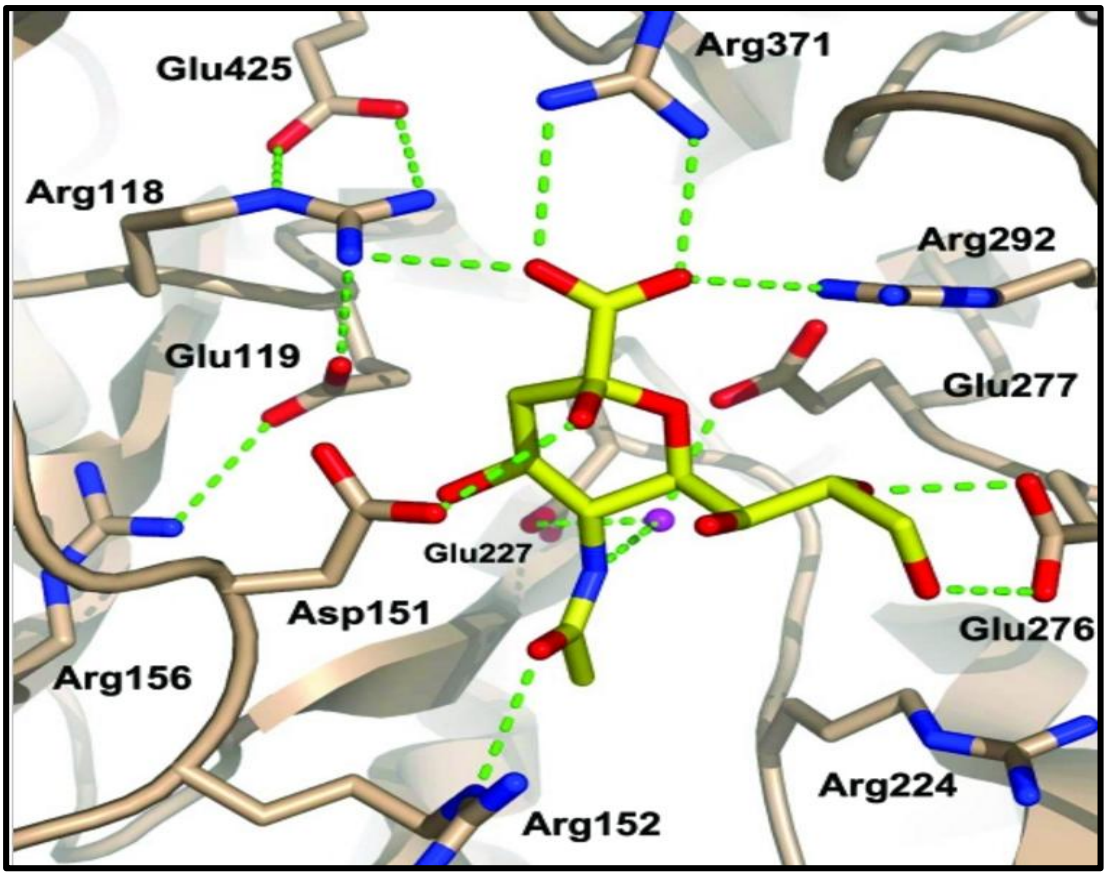


Fig. 1.5 The neuraminidase active site, conserved NA residues (R118, D151, R152, R224, E227, E276, R292, and R371) (Yen *et al*., 2006)

1.9 Natural products

Natural products have many sources, such as microbes, plants, animals, or prebiotic origins (Vuorelaa *et al.*, 2004). Many classes of compounds can be found in natural products. Natural products serve as natural sources of most drugs. Most of the useful therapeutic agents are derived from higher plants. In today's clinical settings,

over 50% of all drugs are based on natural products and their analogs with 25% of such drugs containing higher plant-derived natural products (Balandrin *et al*., 1993).

According to WHO, around 80% of people in developing countries depend on traditional medicine for their daily healthcare (WHO, 2009). Approximately 85% of these medicines are plant extracts; whereby, around 3.5 to 4 billion people worldwide rely on natural plants as drug sources (Farnsworth, 1988). Great efforts have been exerted to discover new antiviral drugs. Many reviews have published discoveries of new natural-based drugs for treating viral infections (Khan *et al*., 2005; Naithani *et al*., 2008). Different reviews have discussed the significant role of traditional medicine in the development and use of antiviral compounds. Traditional medicines such as Chakma medicines and Ayurvedic are interestingly good sources of potential antiviral drugs (Khan *et al*., 2005). Many active compounds from natural sources are used as therapeutics against different functionally diverse and genetic viruses; examples of these compounds include lignans, alkaloids, thiophenes, chlorophyllins, saponins, furyl compounds, limonoids, peptides, and proteins (Chattopadhyay and Bhattacharya, 2008; Naithani *et al*., 2008). The mechanism of anti-viral active compounds can be explained in terms of their scavenging capacities, DNA inhibition, antioxidant activities, RNA synthesis, and inhibition of viral reproduction (Chattopadhyay and Naik, 2007).

According to several pharmacological and chemical studies, some bioactive compounds used to treat influenza are obtained from medicinal plants. Varieties of alkaloids, polyphenols, saponins, and flavonoids isolated from medicinal plants have been extensively tested for anti-influenza activity, which is reflected in the ability of

bioactive compounds to block adherence, duplication, penetration, and maturation during viral propagation (You *et al*., 2013).

Malaysia is home to over 15,000 species of flowering plants and more than 1,100 species of fern allies and ferns, whereby most of the plants are known as endemic plants (Napis *et al*., 2001). A series of chemical structures can be observed in Malaysian flora, especially in secondary flora metabolites. Malaysian plants used as medicine by locals are well documented in books and papers on ethnobotany and ethnopharmacology, with about 1,300 plant species used for medicinal purposes (Napis *et al*., 2001). The traditional medicines available in the markets are mostly in crude form and are sold by traditional medical practitioners. Only 100 species from high-species density available have been systematically evaluated for medicinal properties (Napis *et al*., 2001).

1.9.1 Family of Myrtaceae

The Myrtaceae family known as a large family of more than 5,650 species is organized in 130 to 150 genera. This family is considered as the eighth largest flowering plant family. It has centers of diversity in the wet tropics, particularly South America, Australia, and tropical Asia with occurrences in Africa and Europe (Grattapaglia *et al*., 2010).

The main genera are *Eugenia, Syzygium, Psidium, Pimenta, Myrtus, Pseuocaryophyllus, Plinia, Leptospermum, Melaleuca,* and *Eucalyptus.* Numerous members of this family are used in folk medicine for their antirheumatic, anti-inflammatory, antidiarrheal, antioxidant, and antimicrobial properties (Govaerts *et al*.,

2008). *Syzygium gaertner* is considered as the largest genus of the Myrtaceae family, with approximately 1,200 species distributed in subtropical and tropical regions (Biffin *et al*., 2010). Some promising fruits belonging to the *Syzygium* genus found in Malaysia are under-utilized but are recognized to be useful for medical and nutritional purposes. A number of species from the *Syzygium* genus have been cultivated for their edible, colorful flesh (Rabeta *et al*., 2013).

1.9.1(a) *Psidium guajava*

1.9.1(a)(i) General introduction

P. guajava originated in Mexico (Rios *et al*., 1977) and extends throughout the South America, Europe, Africa, and Asia. Archaeological evidence shows that during pre-Columbian times, this plant was widely used and known in Peru. This plant can adapt to various climate conditions and is found in tropical and subtropical areas worldwide (Stone, 1970). *P. guajava* Linn. (Myrtaceae family) is commonly called guava, goyavier in French, goiabeiro in Portugal, banjiro in Japanese, and guaiaba in Brazil (Killion, 2000). *P. guajava* is known in Malaysian communities as jambu batu and is considered as an important food crop with medicinal use. *P. guajava* is a small, thin tree with a height of 10 m and a smooth peeling bark. It has a short petiolate leaves that are 5-15 cm long. Its flowers have whitish petals measuring up to 20 cm long as well as numerous stamens. The fruit is pear-shaped, small, 3-6 cm long and reddish-yellow when ripe (Stone, 1970). The *P. guajava* plants are shown in Figure 1.6.

Fig. 1.6 *Psidium guajava* plants (a) flower, (b) fruit, (c) leaves and (d) tree (Finca Tropical 2011)

1.9.1(a)(ii) Traditional medicinal uses of *Psidium guajava*

P. guajava was traditionally used as an anti-diarrheal agent and to treat dysentery. Its use as antioxidant, lipid lowering agent, and anti-bacterial agent and its hypoglycemic activities have also been reported (Rahmat *et al*., 2004)**.** *P. guajava* is used in many parts of the world to treat different diseases and conditions, such as fever, pain, wounds, caries, inflammation, and diabetes, as reported in many ethno-pharmacological studies (Table 1.1).

Table 1.1: Ethnomedical uses of *Psidium guajava*.

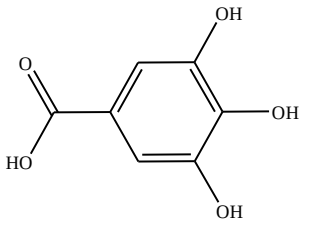
Place, country	Part(s) used	Ethno medical uses	Preparation(s)	Reference
Malaysia	Leaves	Vomiting, diarrhea and stomach ache	Maceration	Alsarhan *et al*., 2010
Panama, Cuba, Costa Rica, M´exico,Nicaragua, Panam´a, Per´u, Venezuela, Mozambique, Guatemala, Argentina	Leaves	anti-inflammatory	Externally applied hot on inflammations	M´endez, 1990; Valdiz´an and Maldonado, 1972
Kinshasa, Congo	Leaves, bark	Diarrhoea, antiamoebic	Infusion or decoction tisane	Tona *et al*. 1999
Latin America, Mozambique	Leaves	Diarrhoea, stomach ache	Infusion or decoction	Pontikis, 1996
South Africa	Leaves	Diabetes mellitus, hypertension	Infusion or decoction	Oh *et al*., 2005; Ojewole, 2005
China	Leaves	Diarrhoea, antiseptic, Diabetes mellitus	Infusion or decoction	Teixeira *et al*., 2003
Brazil	Ripe fruit, flowers, and leaves	Anorexia, cholera, diarrhoea, digestive problems, dysentery, gastric insufficiency, inflamed mucous membranes, laryngitis, mouth (swelling), skin problems, sore throat, ulcers, vaginal discharge	Mashed, Decoction	Holetz *et al*., 2002; Cybele *et al*., 1995

1.9.1(a)(iii) Chemical constituents of *Psidium guajava*

P. guajava contains many phytochemicals including essential oils, polysaccharides, vitamins (Macleod and Troconis, 1975; Smith and Siwatibau, 1975), triterpenoid acid and sesquiterpenoid alcohols (Begum *et al*., 2002; Wilson and Shaw, 1978), saponins, flavonoids , steroids, tannins, glycosides, alkaloids, (Geidam *et al*., 2007; Narayana *et al*., 2001; Cho *et al*., 2003). *P. guajava* is very rich in vitamins and antioxidants and also has a high content of lycopene, lutein and zeaxanthine (Hobert and Tietze, 1998; Tee *et al*., 1997). The *P. guajava* fruit has high water content and low amounts of proteins, fats, and carbohydrates. This fruit also contains thiamine, iron, niacin, manganese, and vitamins A and C. Ascorbic acid is the major constituent of its skin (Gutierrez *et al*., 2008).

Different compounds were isolated from fruits and leave of *P. guajava* such as gallic acid **(1),** protocatechuic acid **(2),** (Okuda *et al.*, 1984) caffeic acid (Liang *et al*., 2005) **(3),** ferulic acid (Zhu *et al*., 1997) **(4),** chlorogenic acid (Liang *et al*., 2005) **(5),** ellagic acid (Misra and Seshadri, 1968) **(6)**, quercetin (Liang *et al*., 2005) **(7)**, leucocyanidin (Nadkarni and Nadkarni, 1999) **(8)**, kaempferol (Liang *et al*., 2005) **(9)**, quercetin 3-β-galactoside (Liang *et al*., 2005) **(10)**, kaempferol 3-β-glucoside (Liang *et al*., 2005) **(11)**, guaijaverin (Prabu *et al*., 2006) **(12)**, morin-3-O-α-L-arabinopyranoside (Arima and Danno, 2002) **(13)**, mecocyanin (Liang *et al*., 2005) **(14)**, quercitrin (Liang et al., 2005) **(15)**, zeatin riboside (Nagar and Rao, 1981) **(16)**, oeanolic acid (Siddiqui *et al*., 2002) **(17)**, ursolic acid (Begum *et al*., 2002) **(18)**, β-sitosterol (Begum *et al*., 2002) **(19)**, uvaol (Begum *et al*., 2004) **(20)**, goreishic acid I (Begum *et al*., 2002) **(21)**, 2-α-hydroxyursolic acid (Begum *et al*., 2002) **(22)**,

guavanoic acid (Begum *et al*., 2004) **(23)** , guavacoumaric acid (Begum *et al*., 2002) **(24)**, guajavolide (Begum *et al*., 2004) **(25)**, 2α-hydroxy-3β-*P*-E-coumaroyloxyurs-12,18-dien-28-oic acid (Begum *et al*., 2002) **(26)**, α-sitosterol-3-O-β-D-glucopyranoside (Begum *et al*., 2002) **(27).**



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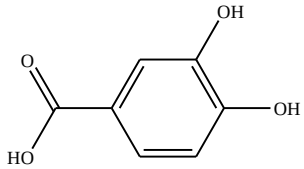

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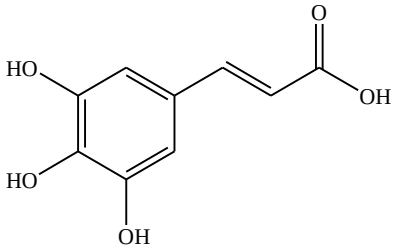

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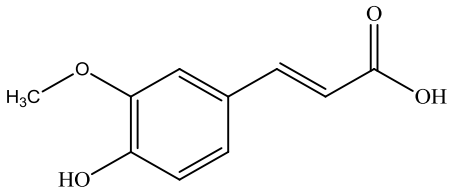

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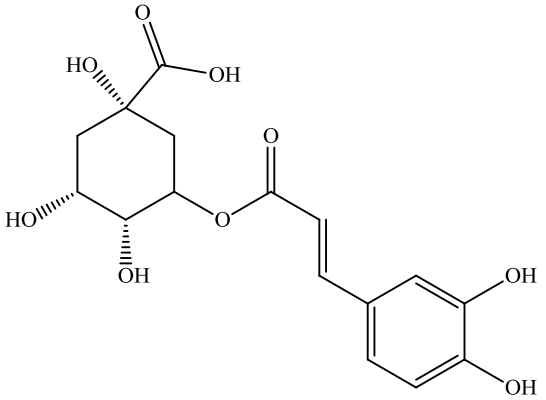

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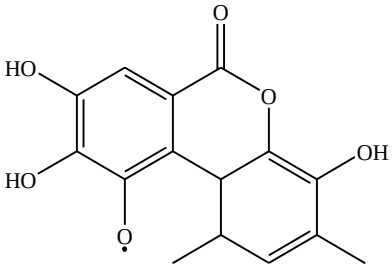

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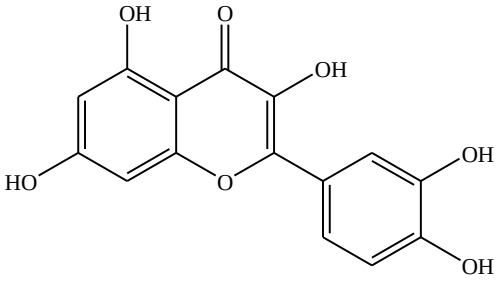
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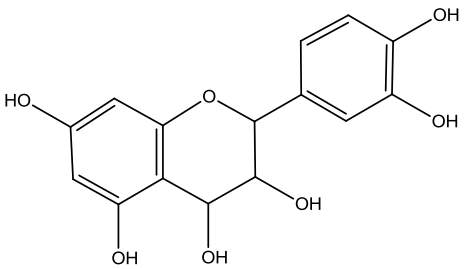
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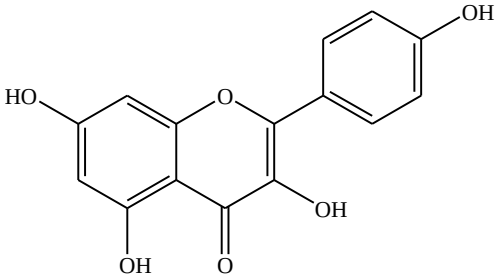
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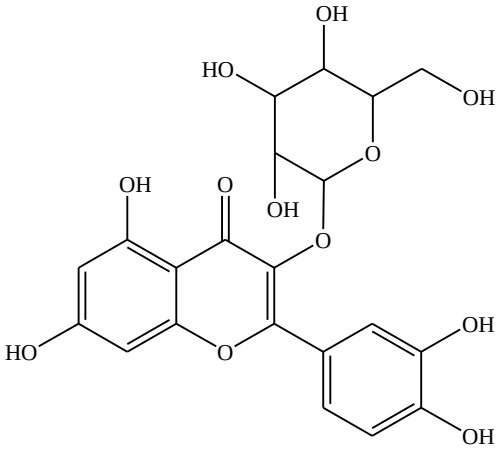
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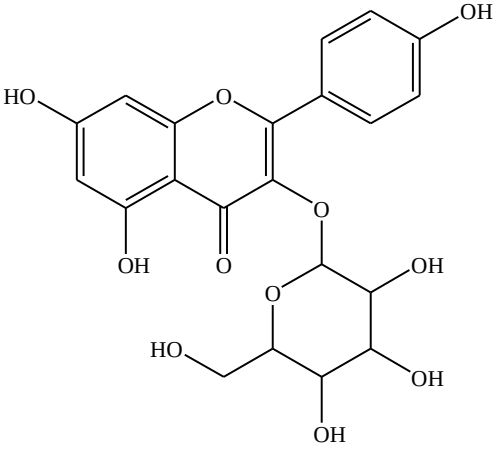
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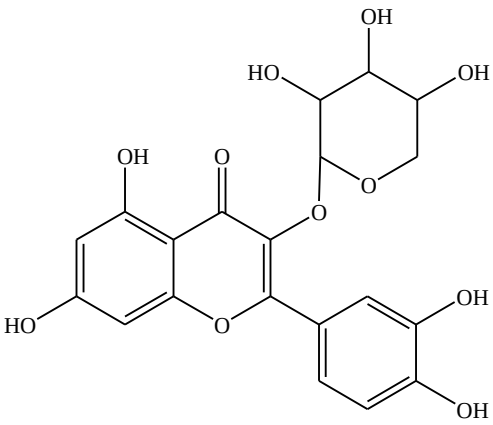

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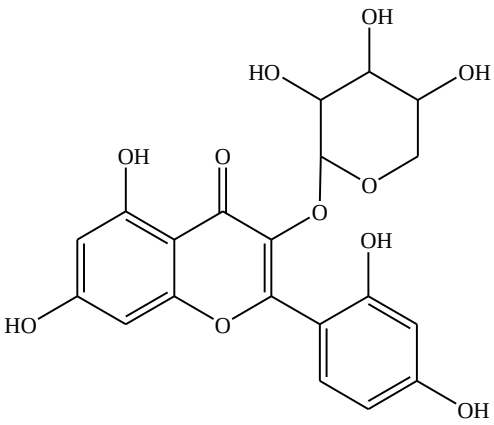

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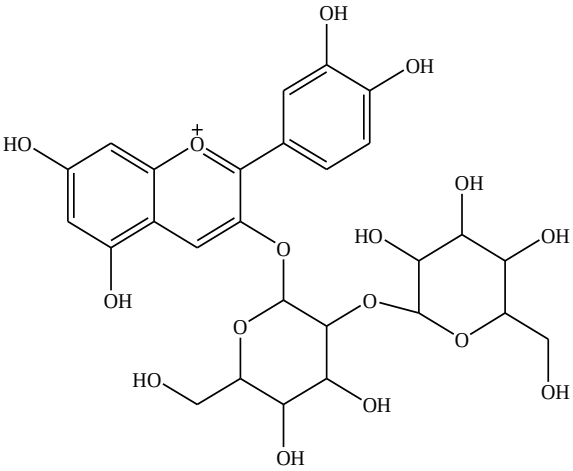

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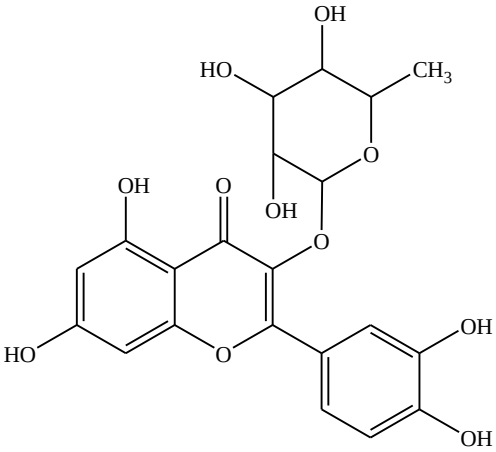

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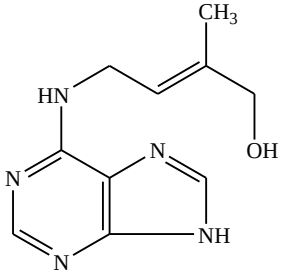

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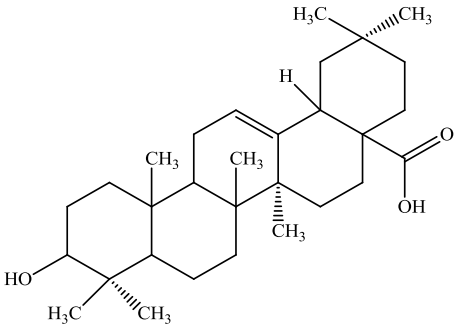

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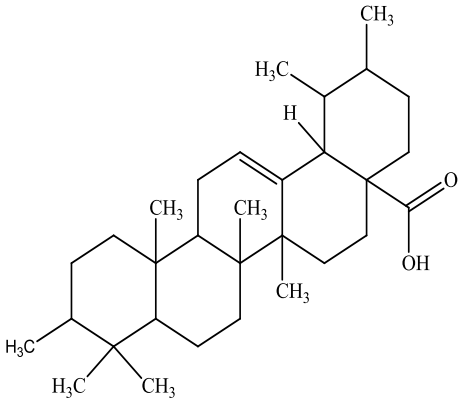

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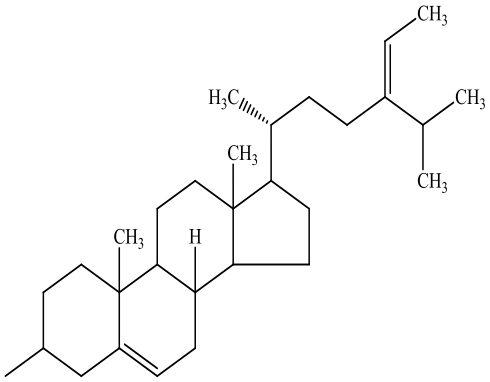

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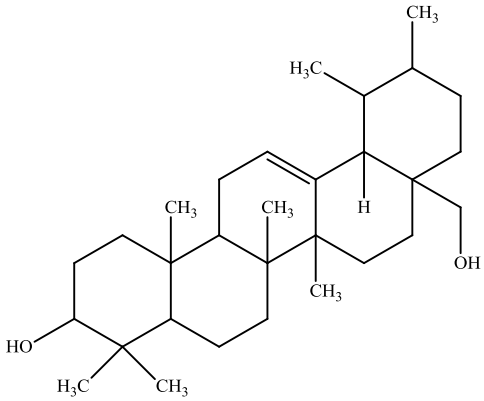

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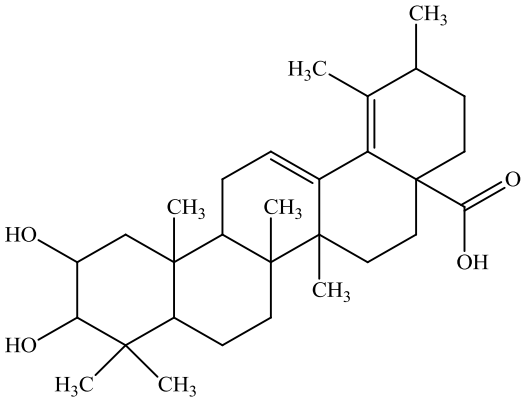

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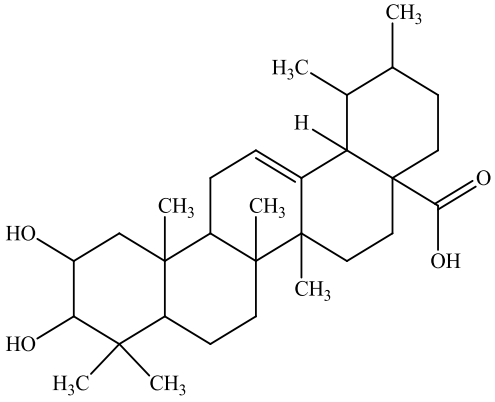

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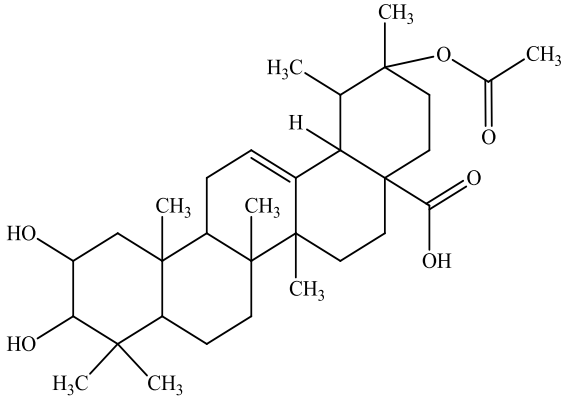

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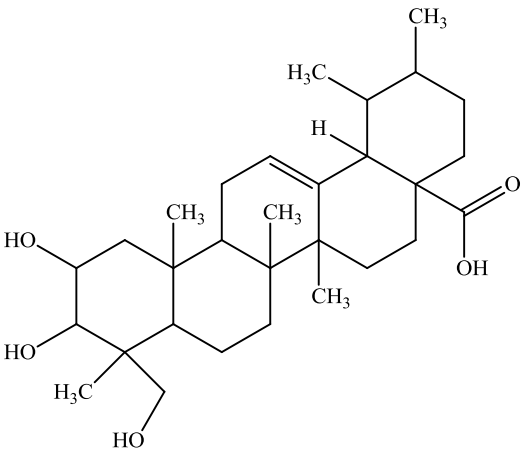
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CH3
CH3
HO
HO
H3C
HO

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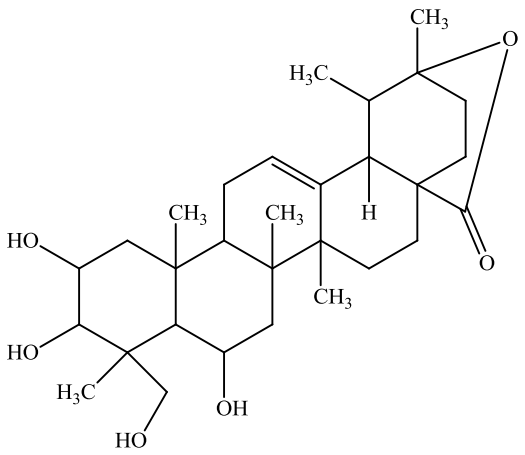
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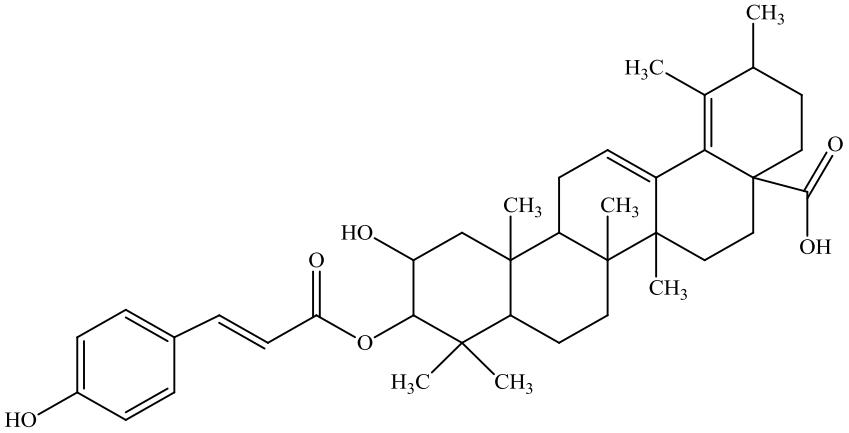
CH3
CH3
HO
HO
H3C
OH
HO

(25)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O
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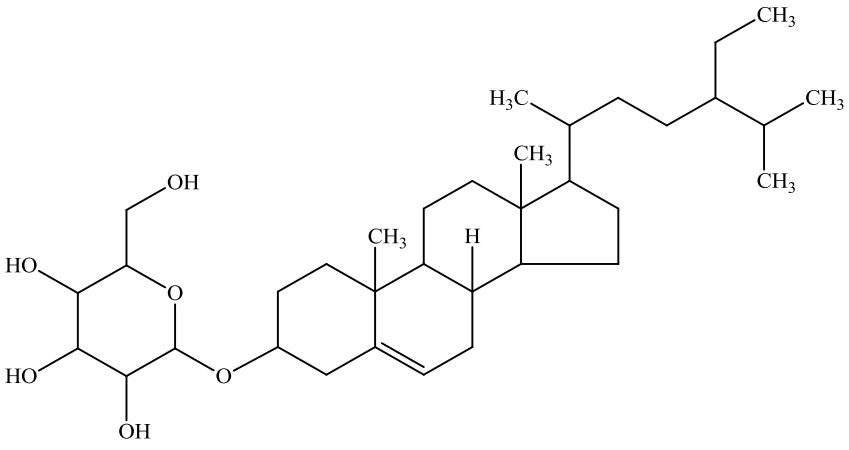
CH3
H3C
CH3
HO

(26)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H3C
CH3
CH3
OH
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H
HO
O
HO
O
HO
OH

(27)

1.9.1(a)(iv) Essential oil of *Psidium guajava*

Analysis of essential oils from *Psidium guajava* (fruit and leaves) using GC and GC-MS enabled the identification different constituents varied from Monoterpenes and Sesquiterpenes. Essential oils are extracted from the fruit, leaves, and seeds of *P. guajava*. The yield and composition of the obtained oils vary from one part to another. Monoterpene hydrocarbon *E*-β-Ocimene, (Li *et al*., 1999) **(28),** *Z*-β-Ocimene (Li *et al*., 1999) **(29),,** Myrcene (Li *et al*., 1999**) (30),** *p*-cymene (Kenneth *et al*., 1970) (**31**),, α-pinene (Kenneth *et al*., 1970) **(32),** β-pinene (Jordan *et al*., 2003) **(33),** camphene (Jordan *et al*., 2003) **(34)**, γ-terpinene (Jordan *et al*., 2003) **(35)** identified from leaves and fruits with different composition. Oxygenated monoterpenes identified from seed and leaves including 1,8-cineole (Kenneth *et al*., 1970) **(36)**, citronellol (Kenneth *et al*., 1970) **(37)** , linalool (Kenneth *et al*., 1970) **(38)**, limonene (Kenneth *et al*., 1970) **(39)** . Sesquiterpenes hydrocarbon from bark, leaves and fruits include α-copaene (Oliver-Bever, 1986) **(40)**, β-copaene (Adam *et al*., 2011) **(41)**, 1-epi-cubenol (Ruther, 2000) **(42)**, α-cubebene (Porat *et al*., 2001) **(43)**, α-selinene (Ruther, 2000) **(44)**, α-Humulene (Paniandy *et al*., 2000) **(45)** β-caryophyllene (Adam *et al*., 2011) **(46)**, γ-cadinene (Ruther, 2000) **(47)**, germacrene D (Oliver-Bever, 1986) **(48)**. Another important constituent oxygenated sesquiterpenes identified with GC-MS from leaves and fruits showed different composition include spathulenol (Porat *et al*., 2001) **(59)**, ar-turmerone (Paniandy *et al*., 2000) **(50)**, δ-cadinol (Ruther, 2000) **(51)**, α-cadinol (Oliver-Bever, 1986) **(52)**, caryophyllene oxide (Ruther, 2000) **(53),** humulene epoxide II (Paniandy *et al*., 2000) **(54)**.